Coordinate Regulation of Mouse Metallothionein I and II Genes by Heavy Metals and Glucocorticoids

MARY K. YAGLE AND RICHARD D. PALMITER*

Howard Hughes Medical Institute Laboratory, Department of Biochemistry, University of Washington, Seattle, Washington 98195

Regulation of the endogenous mouse metallothionein I and II (MT-I and MT-II) genes by heavy metals and glucocorticoids was studied in cultured mouse cells. Both mRNAs were measured simultaneously by solution hybridization with [³H]MT-I cDNA and [³²P]MT-II cDNA, and the absolute amount of each mRNA was calculated by using a single-stranded M13 standard that contained both mRNA sequences. Both genes responded identically to different concentrations of metals (zinc, cadmium, and copper) and dexamethasone. Furthermore, the time courses of induction of both mRNAs were the same. However, under all conditions there was 1.2- to 1.9-fold more MT-I mRNA than MT-II mRNA. We conclude that both genes are regulated identically by receptors for glucocorticoids and metals but that the rate of transcription from the MT-I gene is slightly higher than from the MT-II gene.

Metallothioneins (MTs) are cysteine-rich, heavy-metal-binding proteins found in most animal and plant species. In vertebrates, there are two major forms, designated MT-I and MT-II (10). Both forms are induced by and bind heavy metals such as Zn, Cd, and Cu (7, 10, 17, 20). In mammals, glucocorticoids and inflammatory signals also act as inducers (3, 8, 11). In mice, there is one copy of each gene per haploid genome and they are closely linked on chromosome 8 (2, 24), whereas in primates there are several copies of MT-I and MT-II genes and they reside on several chromosomes (13, 23).

Because the MT isoforms appear to have similar metal binding properties, one reason for the evolution of two similar genes could be to allow differential regulation during development or stress (24). Indeed, the endogenous MT-Ia and MT-IIa genes in cultured human cells have been shown to be differentially regulated by heavy metals and glucocorticoids; the human MT-IIa gene is induced by Cd, Zn, and dexamethasone, whereas the human MT-Ia gene is induced principally by Cd (21). When the respective promoters were fused to the thymidine kinase gene of herpes simplex virus and transferred into rat cells, the corresponding induction phenotypes were observed (21), confirming that the effects are due to differences in the promoter regions.

In contrast to the differential regulation of the human MT-Ia and MT-IIa genes, our results suggested that MT-I and MT-II genes were regulated similarly in intact mice (24). Because it is difficult to control metal and hormone levels in vivo, we proceeded to examine the regulation of these genes in cultured cells. We chose the Hepa 1A cell line to examine metal regulation because the basal level of MT gene expression in this cell line is very low in the absence of added metals. These cells do not respond well to glucocorticoids (14); therefore, mouse L cells were used to study the hormonal response (15). To facilitate quantitation of both mRNAs, we adapted the solution hybridization protocol of Durnam and Palmiter (5) by using differentially labeled cDNAs that were specific to MT-I or MT-II mRNA. In addition, we used a single-stranded M13 phage containing the mRNA strands of both MT-I and MT-II as a standard to allow accurate determination of the absolute ratio of the two mRNAs.

MATERIALS AND METHODS

Quantitation of MT-I and MT-II mRNAs by solution hybridization. Total nucleic acids were harvested by the sodium dodecyl sulfate-proteinase K method and hybridized with ³H- or ³²P-labeled cDNAs essentially as described previously (5). The M13 DNA standards were prepared as described previously (16); the concentration of DNA was accurately determined, and then appropriate dilutions were made in 0.2× SET (1× SET = 1% sodium dodecyl sulfate, 10 mM Tris-hydrochloride, 5 mM EDTA; pH 7.5) containing 100 μg of herring sperm DNA per ml. Total nucleic acids (TNA) prepared from Hepa 1A cells that were resistant to 80 μM Cd were used as an mRNA standard; these cells carry amplified copies of the MT genes (6). The ³H- and ³²P-labeled cDNAs were prepared as described previously (5). About 1,000 cpm of each was combined with the mRNA standards and hybridized at 68°C in 0.6 M NaCl and 40% formamide as described previously (5). The concentration of MT-I and MT-II mRNAs in the RNA standard was determined as shown in Fig. 1; for this experiment the hybridization was performed at 68°C in 30 mM NaCl and no formamide to approximate the same stringency but avoid the preferential stabilization of RNA-DNA hybrids that occurs in formamide. We assume that RNA-DNA and DNA-DNA hybrids form with the same efficiency in the absence of formamide. After 16 h of incubation at 68°C, the samples were treated with S1 nuclease and precipitated with trichloracetic acid, the S1-resistant hybrids were collected on Whatman GF/C filters, and the ³H and ³²P counts were measured with a scintillation counter (5).

RESULTS

Quantitation method for MT-I and MT-II mRNAs. To quantitate mouse MT-I and MT-II mRNAs simultaneously, we cloned a 335-base-pair (bp) RsaI fragment from an MT-I cDNA clone and a 158-bp RsaI fragment from an MT-II cDNA clone into plasmid pBR322. The inserts were then excised and nick translated in the presence of either [³H]TTP (for MT-I) or [³²P]dCTP (for MT-II), and the cDNA strands were isolated (5). MT-I and MT-II inserts were also cloned separately or together into M13 (mp7) to generate single-stranded DNA standards (Fig. 1A). Hybridization of the
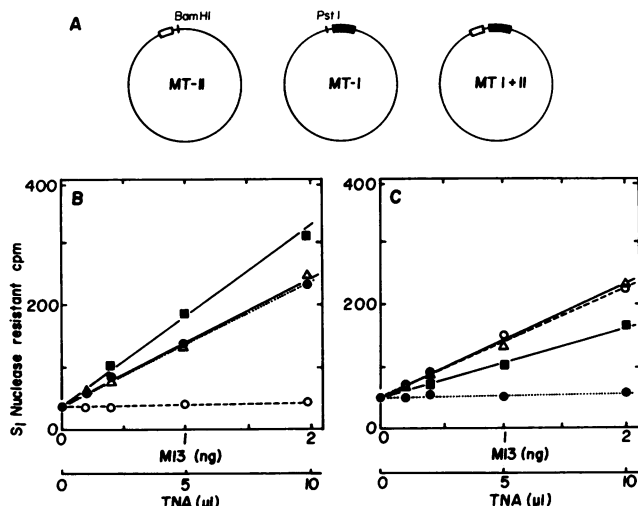
FIG. 1. Quantitation of MT-I and MT-II mRNAs by solution hybridization. A, Diagram of M13 (mp7) standards containing a 158-bp RsaI fragment from MT-II cloned into the PstI site, a 335-bp RsaI fragment from MT-I cloned into the BamHI site, or both. These single-stranded standards (about 7,400 to 7,700 nucleotides) contain the mRNA strand of MT-I or MT-II. B, Hybridization of MT-I [^{3}H]cDNA to the MT-I M13 standard (●), MT-II M13 standard (○), MT-I + II M13 standard (△), or TNA from Cd-resistant Hepa 1A cells (■). C, Hybridization of MT-II [^{32}P]cDNA to the same samples as in B. The experiment shows that ^{3}H-labeled MT-I cDNA hybridizes only to standards containing MT-I sequences and ^{32}P-labeled MT-II cDNA hybridizes only to standards with MT-II sequences. The TNA sample hybridizes to both cDNAs; 5 μl of TNA protects the same amount of MT-I cDNA as 1,400 pg of M13; it also protects the same amount of MT-II cDNA as 600 pg of M13. Thus, the ratio of the two mRNAs in this sample is 1,400/600, or 2.3. Assuming that the length of both mRNAs is about 385 nucleotides, we calculate that 1,400 pg of M13 standard is equivalent to 70 pg of mRNA. Knowing that 5 μl of TNA contains 150 ng of DNA (and 450 ng of RNA) and that there are 19.5 pg of DNA per cell, we calculate that there are 70 pg of MT-I mRNA per 7,700 cells or 42,000 MT-I mRNA molecules per cell.

labeled cDNAs to the M13 standards showed no cross-hybridization at high stringency (30 mM NaCl, 68°C) (Fig. 1B and C). For routine use, we calibrated an RNA sample prepared from cadmium-resistant Hepa 1A cells (Fig. 1B and C) and then used it as a standard in all subsequent hybridization assays, which were performed as described previously (5). From the hybridization results, one can calculate the absolute amount of MT-I and MT-II mRNAs in a sample of TNAs by knowing (i) the DNA concentration, (ii) the amount of DNA per cell, and (iii) the molecular weight of the mRNA (see the legend to Fig. 1).

Response of MT-I and MT-II genes to different concentrations of metals and glucocorticoids. We tested the response of endogenous MT-I and MT-II genes in mouse Hepa 1A cells to a range of concentrations of Zn, Cd, and Cu (Fig. 2A through C). The cells were exposed to the inducer for 8 h before the isolation of nucleic acids. In each case, MT-I and MT-II mRNAs were induced coordinately. Half-maximal and maximal inductions were achieved at the same concentration of metal. However, the average ratio of MT-I to MT-II mRNAs calculated from Fig. 2A through C was 1.7 ± 0.2 (note the 1.4-fold difference in the y axes).

To measure the response of the two MT genes to glucocorticoids, we used mouse L cells because they respond to dexamethasone as well as to Cd (15), whereas the Hepa 1A

cells show very poor response to this synthetic glucocorticoid (14). Once again, both MT-I and MT-II mRNAs were induced coordinately (Fig. 2D). Half-maximal induction was achieved with 15 nM dexamethasone, and the ratio of the two mRNAs was 1.3 ± 0.1 throughout the concentration range tested.

Kinetics of MT mRNA accumulation. The early time course of MT-I and MT-II mRNA accumulation in response to Zn or Cd is shown in Fig. 3A and B. Maximal accumulation of both mRNAs occurred at 6 h, and half-maximal levels were achieved at 2.5 h with either Cd or Zn as the inducing metal. Assuming that metals increase the rate of transcription to a new, constant level, then the time course of mRNA accumulation can be used to deduce the half-life of the mRNA (9). The steady-state equation predicts that the mRNA half-life is equal to the time necessary for half-maximal induction (22). Therefore, the data in Fig. 3A and B suggest that the half-lives of MT-I and MT-II mRNAs are identical (about 2.5 h) with either inducer.

The responses of the two genes to longer exposure to heavy metals were also tested. Fig. 3C and D show the results obtained when Hepa 1A cells were maintained for up to 3 days with 8 μM Cd or 100 μM Zn. We have repeated these experiments many times, and the results typically revealed a peak of mRNA accumulation at the early time point (generally taken at 8 h), followed by a decline during the next 2 days. In some experiments the decline is much more severe than in others; it appears to be more severe with Zn than with Cd, in DME than in F12 media, and with confluent cells than with subconfluent cells. Nevertheless, in all of the experiments the ratio of the two MT mRNAs remained constant.

DISCUSSION

The results presented here indicate that the mouse MT-I and MT-II genes respond identically to three different heavy metals and to dexamethasone. These results agree with the preliminary observations obtained in vivo (24), which indicated that the two mRNAs were coordinately induced in four different tissues by Cd, dexamethasone, or lipopolysaccharide (bacterial endotoxin). The assay used here allows much more precise quantitation. Because both mRNAs were measured in the same sample with a double-isotope protocol and they are present in equal molar amounts in the M13 DNA standard, error in their quantitation is minimal. Although the two mRNAs were induced coordinately, there was consistently 1.2- to 1.9-fold more MT-I mRNA than MT-II mRNA in both the Hepa 1A and the L cells. We also measured the ratio of these two mRNAs in liver samples from mice treated with zinc and found the ratio of MT-I to MT-II mRNA to be 1.5 ± 0.35 (n = 9). The preferential accumulation of MT-I mRNA therefore appears to be a general property in vivo as well as in culture. The ratio of the two mRNAs is also consistent with the ratio of the two proteins in Hepa 1A cells (6) and various mouse and rat tissues (see reference 25 for review).

We assume that regulation of MT genes is mediated by a metal-binding regulatory protein(s). The identical responses of the two MT genes to three different metals is most easily explained by the interaction of the same regulatory protein(s) with the promoter elements of these genes. With this interpretation, the dose-response curves reflect the occupancy of the postulated regulatory protein(s) with metal. Analysis of DNA sequences in the vicinity of the MT gene promoters reveals several conserved elements, some of which have been implicated in metal regulation (12, 24; G. W. Stuart,
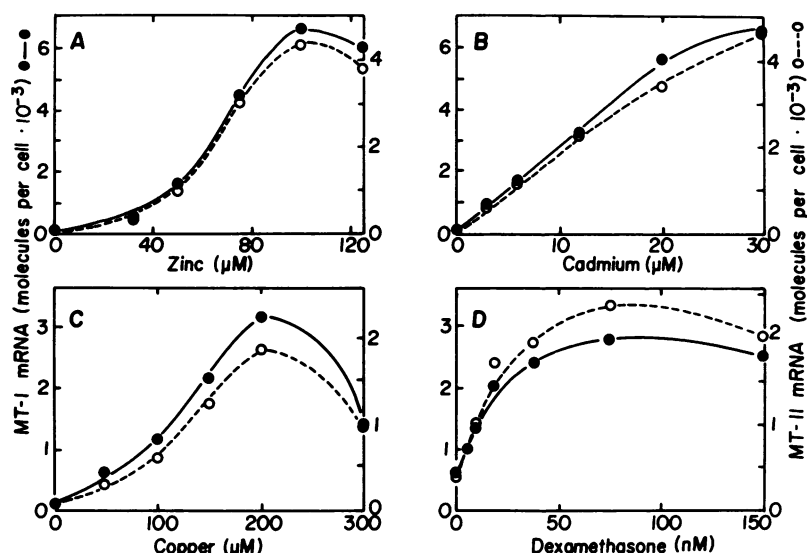
FIG. 2. Induction of MT-I and MT-II mRNA by metals and dexamethasone. A through C, Hepa 1A cells were plated in Dulbecco modified Eagle medium containing 10% fetal calf serum and the indicated concentrations of Zn, Cd, or Cu, respectively. D, Mouse L cells were plated in the same medium containing the indicated concentrations of dexamethasone, a synthetic glucocorticoid. In each case, 8 h after the inducer was added, the cells were washed in saline and then TNA were isolated by the sodium dodecyl sulfate-proteinase K method (5). Three different portions of nucleic acid were hybridized with ^{3}H-labeled MT-I cDNA and ^{32}P-labeled MT-II cDNA by using the standard conditions described previously (5) and the TNA standard that was calibrated as shown in Fig. 1. The number of molecules of MT-I and MT-II mRNA per cell was calculated (as indicated in the legend to Fig. 1) by assuming 12.6 pg of DNA per cell for Hepa 1A and L cells.

P. F. Searle, H. Y. Chen, R. L. Brinster, and R. D. Palmiter, Proc. Natl. Acad. Sci. U.S.A., in press). The sequence element responsible for metal regulation of the MT-I gene is repeated several times, with minor sequence variation, between −40 and −180 bp; these elements appear to act cooperatively to allow maximal induction (Stuart et al., in press). A similar array of related sequences is present.
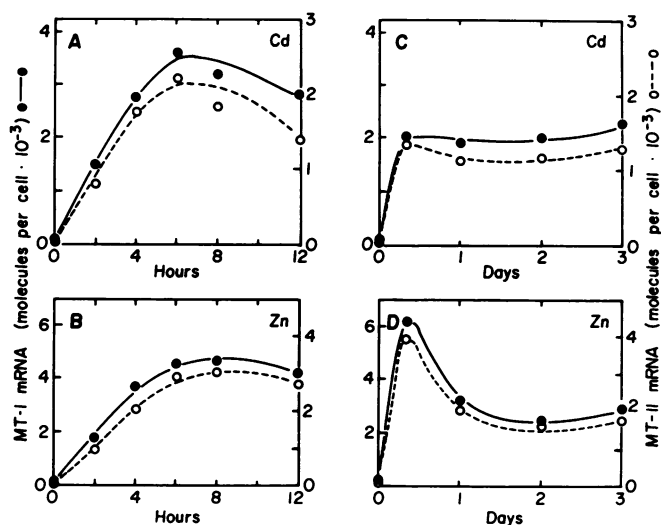

FIG. 3. Kinetics of MT-I and MT-II mRNA accumulation in response to zinc and cadmium. Hepa 1A cells were plated in Dulbecco modified Eagle medium containing 10% fetal calf serum. A and C, 8 μM CdSO₄ was added and the cells were harvested at the indicated times; B and D, 100 μM ZnSO₄ was added to the medium. In C and D, different numbers of cells were plated such that the cells would be at about the same cell density (subconfluent) at the time of harvest. Nucleic acid samples were prepared and hybridized as described in the text.

between −40 and −300 bp of the mouse MT-II gene (24). Although there are variations in the distribution and exact sequence of the elements in the two promoters, the results presented here suggest that they are functionally equivalent.

Karin et al. (12) have located a glucocorticoid receptor binding site at about −250 bp in the human MT-IIa promoter. However, we have been unable to locate the sequences involved in glucocorticoid regulation in either of the mouse MT genes because the response to these hormones is lost on gene transfer (19, 24). Nevertheless, the results presented here argue that the glucocorticoid receptor binding sites are functionally equivalent in mouse MT-I and MT-II genes.

The observation that the ratio of MT-I to MT-II mRNA is consistently about 1.5 probably reflects a correspondingly higher rate of transcription from the MT-I gene. The ratio of the two mRNAs could be due to differences in either transcription or stability; however, there is ample evidence to suggest that these genes are transcriptionally controlled (1, 4, 8, 12), and the kinetics of MT-I and MT-II mRNA accumulation (Fig. 3) suggest that both mRNAs have the same half-life (about 2.5 h). The fact that this ratio is maintained with different inducers could be explained either by a higher affinity of metal and glucocorticoid receptors for MT-I promoter sequences compared with MT-II sequences or by assuming that some other element in the MT-I promoter allows it to respond slightly more efficiently to either inducer.

MT gene promoters have been fused to various structural genes to allow their expression to be regulated (1, 12, 18, 19, 21). Under some circumstances, one might like to control the steady-state concentration of the fusion gene product by manipulating the rate of transcription. The long-term kinetics (Fig. 3C) suggest that this may be feasible; however, our experience suggests that other factors can influence the steady-state level of mRNA in a rapidly growing cell population. In particular, we frequently observed a decline in MT

mRNA levels after an initial peak at 6 to 8 h (Fig. 3D). The concentration of Zn which allows optimal induction of MT mRNA is compatible with continued cell growth, whereas the optimal concentration of Cd is not. Therefore, it is not possible to maintain a high level of MT mRNA with Cd alone, although at suboptimal concentrations a relatively steady level of MT mRNA can be maintained (Fig. 3C). The presence of Zn counteracts some of the toxic effects of Cd, an observation that prompted us to try combinations of the two metals. Initial experiments suggest that this may be a means of maintaining a high, steady level of MT mRNA and rapid cell growth.

The results presented here are in striking contrast to those obtained by Richards et al. (21), who obtained different responses of the human MT-Ia and MT-IIa genes to Zn and Cd as well as to dexamethasone. Furthermore, these differences persisted after transfer of fusion genes into rat cells. Their results suggest that the promoter regions of human MT genes have diverged so that they bind regulatory proteins differentially, whereas in mice the promoter regions of the two MT genes are functionally similar. Analysis of the regulation of MT genes from other species is necessary to piece together the evolution of their regulatory mechanisms. This approach may also provide insight into the functions of these ubiquitous proteins.

ACKNOWLEDGMENTS

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